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Activation of the AT_1 Angiotensin Receptor Is Dependent on Adjacent Apolar Residues in the Carboxyl Terminus of the Third Cytoplasmic Loop*

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The C-terminal region of the third intracellular loop of the AT_1 angiotensin receptor (AT_1-R) is an important determinant of G protein coupling. The roles of individual residues in agonist-induced activation of $G_{q/11}$-dependent phosphoinositide hydrolysis were determined by mutational analysis of the amino acids in this region. Functional studies on mutant receptors transiently expressed in COS-7 cells showed that alanine substitutions of the amino acids in positions 232–240 of the third loop had no major effect on signal generation. However, deletion mutations that removed Ile^{238} or affected its position relative to transmembrane helix VI significantly impaired angiotensin II-induced inositol phosphate responses. Substitution of Ile^{238} with an acidic residue abolished the ability of the receptor to mediate inositol phosphate production, whereas its replacement with basic or polar residues reduced the amplitude of inositol phosphate responses. Substitutions of Phe^{239} with polar residues had relatively minor effects on inositol phosphate signal generation, but its replacement by aspartic acid reduced, and by positively charged residues (Lys, Arg) significantly increased, angiotensin II-induced inositol phosphate responses. The internalization kinetics of the Ile^{238} and Phe^{239} mutant receptors were impaired in parallel with the reduction in their signaling responses. These findings have identified Ile^{238} and Phe^{239} as the critical residues in the C-terminal region of the third intracellular loop of the AT_1-R for receptor activation. They also suggest that an apolar amino acid corresponding to Ile^{238} of the AT_1-R is a general requirement for activation of other G protein-coupled receptors by their agonist ligands.

The AT_1 angiotensin receptor (AT_1-R)[1] is a member of the GPCR superfamily and mediates the physiological actions of the octapeptide hormone, Ang II, on cardiovascular regulation and salt/water balance. The binding of Ang II to the AT_1-R initiates conformational changes that lead to activation of its cognate G protein(s), predominantly $G_{q/11}$, in numerous Ang II target tissues. The subsequent stimulation of phospholipase C causes the generation of inositol 1,4,5-trisphosphate and diacylglycerol, leading to the elevation of $[Ca^{2+}]$ and activation of protein kinase C (1–3). The AT_1-R is also coupled to other signaling pathways, including the Ras-mediated activation of mitogen-activated protein kinase and cell growth responses (4, 5).

Although GPCRs exhibit a remarkable degree of structural and functional diversity, all share a common architecture in which seven transmembrane helices are linked by alternating intracellular and extracellular loops. Agonist binding to the extracellular loops, and/or the exofacial regions of the transmembrane domains, induces conformational changes in the transmembrane helices and intracellular regions that promote coupling of the activated receptor to its cognate G protein(s). The receptor domains that are involved in G protein coupling have been analyzed in many GPCRs. Studies using chimeric α_2/β_2-, α_{1B}/α_2-, and α_1/β_2-adrenergic receptors and chimeric M_2/M_3 muscarinic receptors have shown that modification of IL3 influences G protein selectivity, suggesting that IL3 has a critical role in receptor-G protein interaction (6–9). The importance of the C-terminal portion of IL3 in receptor conformation has been further indicated by the ability of mutations of specific residues in this region to cause constitutive activation of several adrenergic receptors (6–8).

Studies on spin-labeled double cysteine mutants of rhodopsin have indicated that rigid body motion of helix VI compared with helix VII is an important event in receptor activation (10) and that movement of the C-terminal region of IL3 occurs in constitutively active receptors (11). In the β_2-adrenergic receptor, substitution of the C-terminal region of IL3 with those of the α_{1B}- or α_{2A}-adrenergic receptors abolished the ability to couple to G_s (9, 12). In M_2, M_3, and M_5 muscarinic receptors, this region has been implicated in agonist-induced coupling to $G_{q/11}$ or G_i, respectively (13–15). Studies on chimeric human AT_1- and AT_2-Rs have demonstrated that the membrane proximal portions of IL3 are important for coupling to G_q (16). Also, the substitution of all charged residues in the C-terminal region of IL3 markedly reduces Ang II-induced inositol phosphate signal generation (17). Furthermore, exchange of this region of the AT_1-R with the corresponding sequence of the β_2-adrenergic receptor caused coupling of the receptor to G_s as well as G_q during agonist stimulation (18). Other studies have demonstrated that a 16-amino acid peptide comprising the

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[1] The abbreviations used are: AT_1-R, type 1 angiotensin receptor; GPCR, G protein-coupled receptor; Ang II, angiotensin II; IL2, second intracellular loop; IL3, third intracellular loop; GTPγS, guanosine 5′-*O*-(thiotriphosphate); DMEM, Dulbecco's modified Eagle's medium; K_e, endocytotic rate constant; Sar, sarcosine.

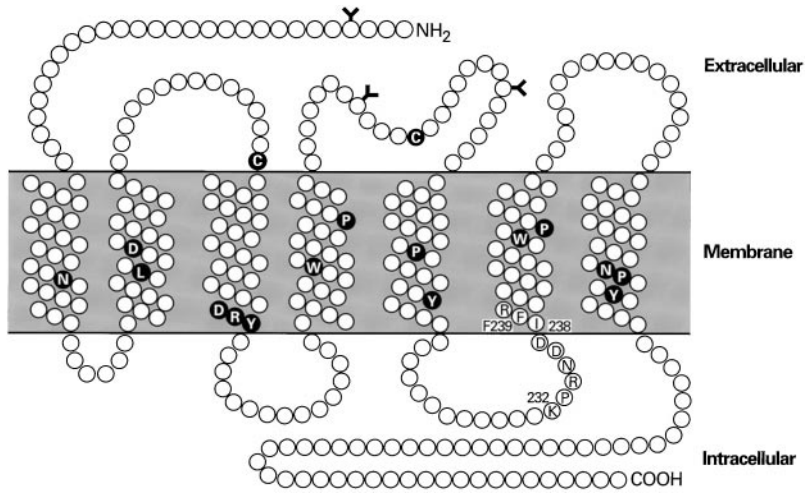

FIG. 1. **Structure of the rat AT_{1a}-R.** The positions of the amino acids are based on the model of Baldwin (40). The most conserved residues in GPCRs are shown in *white letters* on a *black* background. Glycosylation sites of the receptor and amino acids in the third intracellular loop of the AT_1-R that were mutated in this study are also indicated.

N-terminal portion of IL3 of the AT_1-R stimulates the binding of GTPγS to G_q (19).

Although these findings have clearly implicated the C-terminal region of IL3 in the coupling of the AT_1-R to G_q, the amino acids that are critical for this interaction have not been identified. To resolve this question, agonist-induced functional responses of mutant AT_{1a}-Rs bearing replacements and deletions of amino acids in this region were determined in transiently transfected COS-7 cells.

EXPERIMENTAL PROCEDURES

Materials—DMEM, fetal bovine serum, and antibiotic solutions were from Biofluids (Rockville, MD). Ang II was from Sigma. ^{125}I-Ang II and [^{125}I-Sar^1,Ile^8]Ang II were from Covance Laboratories (Vienna, VA) or NEN Life Science Products. *myo*-[2-^{3}H]Inositol was from Amersham Pharmacia Biotech. OptiMEM, inositol-free DMEM, and LipofectAMINE were from Life Technologies, Inc.

Mutagenesis and Transient Expression of Mutant Rat AT_{1a}-Rs—Mutations were created using the Mutagene kit (Bio-Rad) in the sequence of the rat AT_{1a}-R cDNA subcloned into the mammalian expression vector, pcDNAI/Amp (Invitrogen, San Diego, CA), and mutated sequences were verified by dideoxy sequencing as described previously (20). Rat AT_{1a} replacement mutant receptors are indicated by the single letter code of the original amino acid followed by the position number and the single letter code of the exchanged amino acid. Deletion mutant AT_{1a} receptors are indicated by Del followed by the single letter code of the deleted amino acid and its position number. COS-7 cells were seeded at 5×10^4 cells/well in 24-well culture plates in 1 ml of DMEM containing 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (COS-7 medium) and cultured for 3 days before transfection using 0.5 ml of OptiMEM containing 8 μg/ml of LipofectAMINE and the required DNA (usually 2 μg/ml) for 6 h at 37 °C. Cells were cultured for 2 additional days in fresh COS-7 medium prior to use.

[Sar^1,Ile^8]Ang II Binding to Intact Cells—To determine the expression level and structural integrity of the mutant receptor, the number of Ang II binding sites was determined by incubating the transfected cells with [^{125}I-Sar^1,Ile^8]Ang II (0.05–0.1 μCi/sample) and increasing concentrations of unlabeled [Sar^1,Ile^8]Ang II in DMEM for 6 h at 4 °C. The bound radioactivity associated with the cells was measured by γ-spectrometry, and the binding curves were analyzed with the LIGAND computer program using a one-site model as described previously (21).

AT_{1a}-R Internalization Assay—Internalization of mutant and wild-type AT_{1a}-Rs were measured using ^{125}I-Ang II at 37 °C by γ-spectrometry, and values of the endocytotic rate constants (K_e) were calculated as described previously (21). The percent of internalized ligand at each time point was calculated from the ratio of the acid-resistant specific binding to the total (acid-released + acid-resistant) specific binding.

Inositol Phosphate Measurements—Transfected COS-7 cells in 24-well plates were labeled by incubation for 24 h in inositol-free DMEM containing 0.1% (w/v) bovine serum albumin, 2.5% (v/v) fetal bovine serum, antibiotics, and 20 μCi/ml *myo*-[2-^{3}H]inositol. After washing and preincubation with 10 mM LiCl for 30 min, 1 μM Ang II was added for a further 20 min, and incubations were stopped with perchloric acid. Inositol phosphates were extracted, the combined inositol bisphosphates + inositol trisphosphates fractions were separated using Bio-Rad AG 1-X8 columns, and their radioactivities were determined by liquid scintillation counting as described (22). At the expression levels used in this study, there was a linear relationship between cell surface receptor expression and the magnitude of agonist-stimulated inositol phosphate production (21).

RESULTS

Binding Properties of Mutant AT_1-Rs—To identify residue(s) in the C-terminal region of IL3 of the AT_{1a}-R that are required for receptor activation, we analyzed a series of mutant receptors containing replacements or deletions of residues in the Lys^{232}-Phe^{239} segment (Fig. 1). All mutants retained high binding affinity for [^{125}I-Sar^1,Ile^8]Ang II (Table I), indicating their retention of the normal global conformation of the wild-type receptor. However, the mutant receptors displayed considerable variation in their expression levels compared with the wild-type receptor, and the expression of the Del N235 mutant receptor was markedly reduced (Table I).

Inositol Phosphate Responses of Alanine Replacement and Deletion Mutant AT_1-Rs—The ability of each mutant receptor to couple to G_q was evaluated by measuring the Ang II-induced stimulation of inositol phosphate production in transfected COS-7 cells. Alanine substitutions of the AT_{1a}-R caused no major changes of the basal inositol phosphate levels (Fig. 2). The Ang II-stimulated inositol phosphate responses were significantly reduced in the D236A and D237A mutant receptor (Fig. 2, *upper panel*), but the expression levels of these mutant receptors were also lower (Table I). When the inositol phosphate responses were normalized to the number of expressed receptors, it was evident that the replacement of each residue in the Lys^{232}-Phe^{239} segment by alanine had no significant effect on the ability of the receptor to couple to G_q and initiate signal transduction (Fig. 2, *lower panel*). Double alanine substitution of Lys^{232} and Arg^{234} had no major effect on receptor expression (Table I) or inositol phosphate signal generation (Fig. 2). Triple alanine substitution of Asn^{235}, Asp^{236}, and Asp^{237} markedly decreased the expression level (Table I) and the InsP responses of this mutant receptor (data not shown). The reduced inositol phosphate responses observed after triple alanine substitution of Ile^{238}, Phe^{239}, and Arg^{240} (Fig. 2, *upper panel*) could be attributed to the decreased expression level of this mutant receptor (Fig. 2, *lower panel*, Table I). These data indicate that negatively charged amino acids in this region are required for normal receptor expression and argue against the role of charged residues in inositol phosphate signal generation.

The C terminus of IL3 of GPCRs has been predicted to

TABLE I

Binding parameters of mutant rat AT_{1a}-Rs for [^{125}I-Sar^1,Ile^8]Ang II

K_d and B_{max} values were calculated using the LIGAND program. The numbers of expressed binding sites are shown as a percent of the binding sites of the wild-type AT_{1a}-Rs measured in the same experiment. The expression level of the wild-type AT_{1a}-R was 2.48 ± 0.76 pmol/mg of protein. The data are expressed as mean ± S.E. of three independent experiments each performed in duplicate.

AT_{1a}-R	K_d	B_{max}
	nM	*(% of wild-type)*
Wild-type	1.75 ± 0.24	100.0
K232A	2.35 ± 0.50	117.4 ± 24.1
P233A	1.88 ± 0.50	91.1 ± 5.2
R234A	1.71 ± 0.39	53.1 ± 1.0
N235A	1.74 ± 0.18	44.7 ± 2.4
D236A	2.13 ± 0.65	23.5 ± 1.5
D237A	2.96 ± 0.48	17.1 ± 0.1
K232A/R234A	1.52 ± 0.41	110.4 ± 5.9
N235A/D236A/D237A	1.38 ± 0.35	5.3 ± 1.97
I238A/F239A/R240A	1.47 ± 0.44	12.1 ± 0.31
I238A	1.45 ± 0.11	128.0 ± 5.7
I238S	1.55 ± 0.15	146.3 ± 15.4
I238K	1.60 ± 0.18	99.6 ± 5.2
I238D	1.80 ± 0.22	240.0 ± 4.4
F239A	1.20 ± 0.06	28.6 ± 2.7
F239L	1.66 ± 0.48	138.2 ± 36.6
F239N	1.12 ± 0.22	126.7 ± 45.1
F239S	1.94 ± 0.15	87.8 ± 1.8
F239Y	1.21 ± 0.17	75.3 ± 24.1
F239K	0.96 ± 0.24	31.3 ± 0.3
F239R	2.55 ± 0.07	36.3 ± 0.8
F239D	1.56 ± 0.04	91.6 ± 0.3
Del P233	1.69 ± 0.56	103.3 ± 10.3
Del R234	2.05 ± 0.58	98.5 ± 2.1
Del N235	1.53 ± 0.06	0.4 ± 0.2
Del D237	1.28 ± 0.26	44.6 ± 3.2
Del I238	1.84 ± 0.39	139.8 ± 6.2
Del F239	1.88 ± 0.14	48.0 ± 3.6
Del R240	4.43 ± 1.06	30.0 ± 0.3

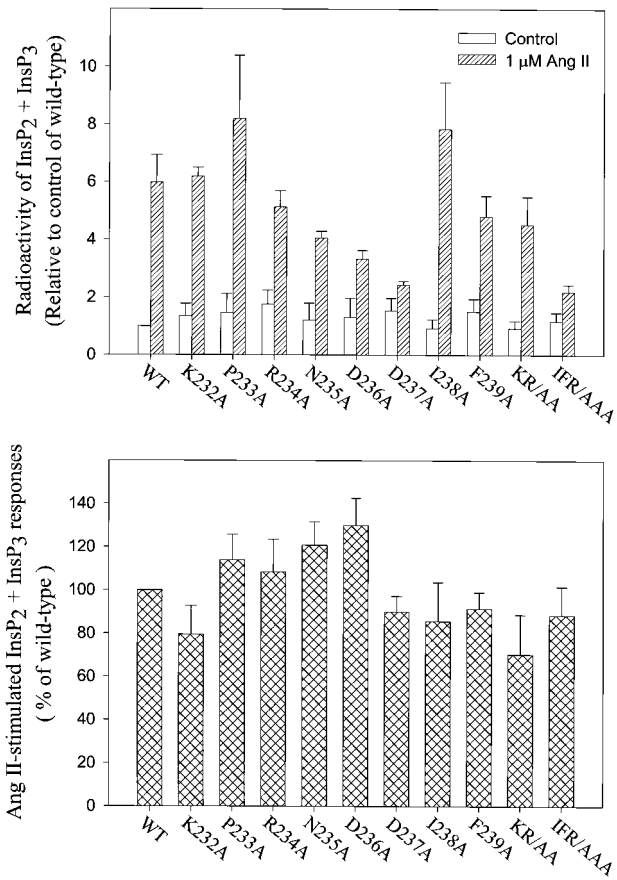

FIG. 2. **Inositol phosphate responses of alanine replacement mutant AT_{1a}-Rs.** *Upper panel,* [^{3}H]inositol-labeled COS-7 cells expressing the wild-type (*WT*), the indicated point mutant, the K232A/R234A (*KR/AA*) double alanine substitution mutant, or the I238A/F239A/R240A (*IFR/AAA*) triple alanine substitution mutant AT_{1a} receptors were preincubated with 10 mM LiCl for 30 min prior to the addition of Ang II. After incubation for 20 min in the absence (*open bars*) or presence of 1 μM Ang II (*hatched bars*) the radioactivity of [^{3}H]inositol phosphates was measured as described under "Experimental Procedures." *Lower panel,* inositol phosphate responses were normalized to the number of expressed receptors (Table I) and are shown as a percentage of the wild-type response. The data represent mean ± S.E. from three independent experiments each performed in duplicate. *$InsP_2$*, inositol bisphosphates; *$InsP_3$*, inositol trisphosphates.

assume an α-helical structure that is continuous with the sixth transmembrane helix (23). Because deletions of individual residues within a helix change the orientation of the remaining amino acids, such mutations are likely to modify or abolish functionally important contacts by the constituent residues of the helix. When single amino acids in the C-terminal region of IL3 were deleted to identify functionally important residues, only the removal of Asn^{235} reduced the expression of the AT_{1a}-R to such an extent that normalization of its inositol phosphate response was not reliable. Deletion of residues Arg^{240}, Phe^{239}, and Ile^{238}, adjacent to the sixth intramembrane helix, markedly impaired Ang II-induced inositol phosphate generation (Fig. 3). However, deletion of Asp^{237}, Arg^{234}, or Pro^{233}, residues that are more distant from helix 6, had no effect on the inositol phosphate responses (Fig. 3). These findings suggest that the residue located in position 238 is an important structural determinant of Ang II-induced signal generation.

Inositol Phosphate Responses of Ile^{238} and Phe^{239} Substitution Mutant AT_1-Rs—In adrenergic receptors, substitutions of amino acids in the location corresponding to position 239 of the AT_{1a}-R cause constitutive activation of the mutant receptors (24). A more detailed analysis of the structural requirements in positions 238 and 239 was performed to study the role of this region in AT_{1a}-R activation. Replacement of Phe^{239} with other apolar residues such as alanine and leucine or with tyrosine, a polar residue, had little or no effect on inositol phosphate signal generation (Fig. 4). However replacement with asparagine or serine significantly impaired the signaling ability of the AT_1-R. Replacement of Phe^{239} with the acidic residue, aspartate, had the most profound effect and reduced inositol phosphate production by about 90%. In contrast, replacement of Phe^{239} with basic residues (lysine or arginine) significantly enhanced inositol phosphate responses. None of the tested mutants had a major effect on inositol phosphate levels in unstimulated cells, indicating that, in contrast to the adrenergic receptor(s), replacement of the amino acid in this position with other residues does not cause constitutive activation of the AT_{1a}-R (Fig. 4, Table I).

Replacement of Ile^{238} with polar or charged amino acids also affected the signal generation efficacy of the AT_1-R (Fig. 5). Substitution of Ile^{238} with apolar alanine had no effect on inositol phosphate responses, but replacement with polar serine impaired inositol phosphate production when the data were normalized to the expression levels of the receptor (Fig. 5, *lower panel*). Replacement of Ile^{238} with aspartate considerably increased the expression of the receptor in COS-7 cells (Table I). However, despite the increased expression level of the I238D receptor its inositol phosphate responses were significantly impaired and when normalized for expression were reduced by ~85%. Substitution of Ile^{238} with lysine, a basic residue, had no effect on the expression level of the receptor, but the inositol phosphate responses of the I238K receptor were significantly reduced (Fig. 5, Table I).

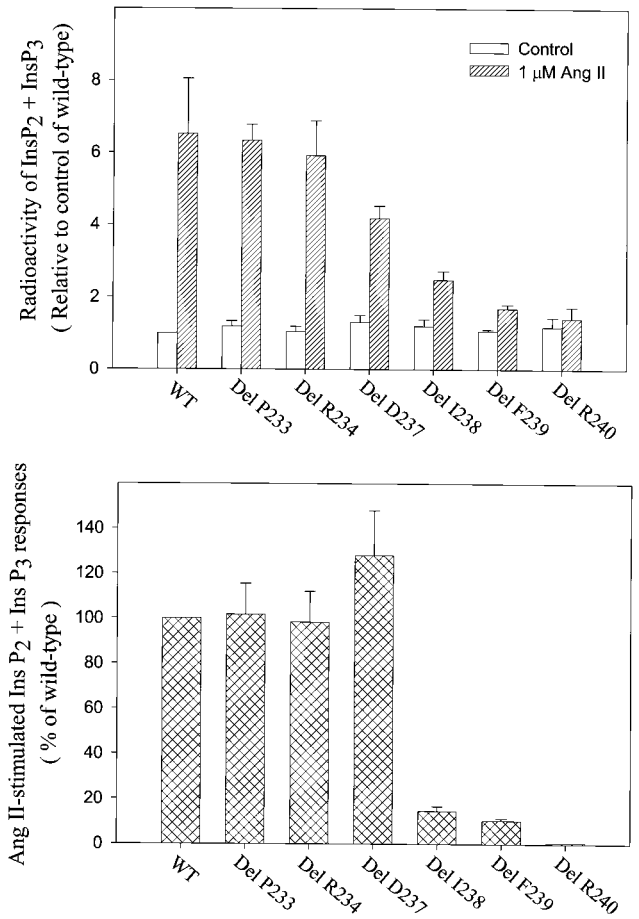

FIG. 3. **Inositol phosphate responses of single deletion mutant rat AT_{1a}-Rs.** Radioactivity of inositol bis- (*$InsP_2$*) and trisphosphates (*$InsP_3$*) (*upper panel*) and normalized inositol phosphate responses (*lower panel*) of cells expressing wild-type (*WT*) and mutant rat AT_{1a}-Rs are shown as described in the legend to Fig. 2. The data represent mean ± S.E. from three independent experiments each performed in duplicate.

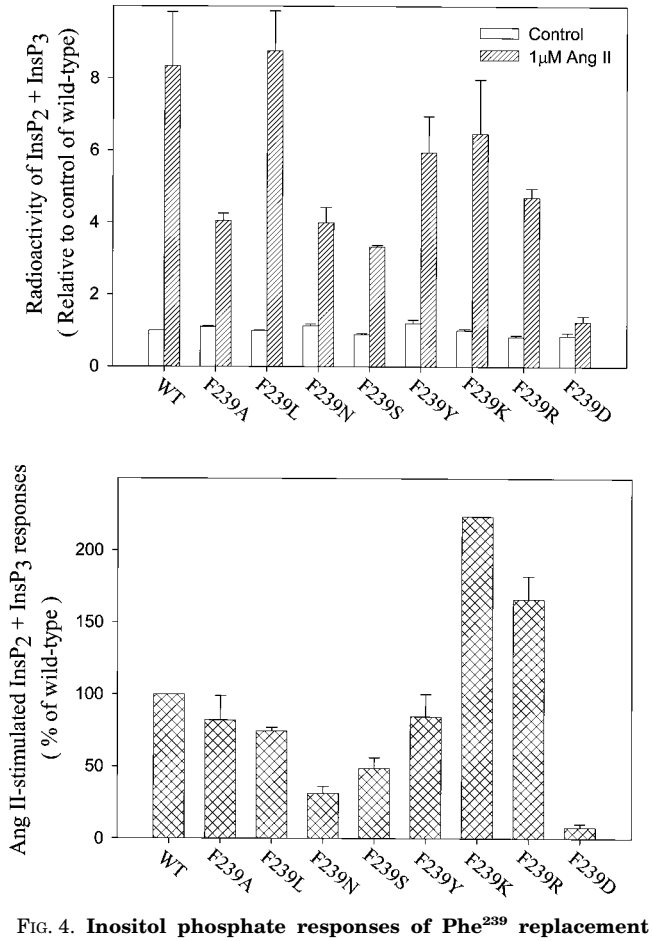

FIG. 4. **Inositol phosphate responses of Phe^{239} replacement mutant AT_{1a}-Rs.** Radioactivity of inositol bis- (*$InsP_2$*) and trisphosphates (*$InsP_3$*) (*upper panel*) and normalized inositol phosphate responses (*lower panel*) of cells expressing wild-type (*WT*) and mutant rat AT_{1a}-Rs are shown as described in the legend to Fig. 2. The data represent mean ± S.E. from three independent experiments each performed in duplicate.

Internalization of Mutant AT_{1a}-Rs—Individual replacements of each amino acid in the Lys^{232}-Ile^{238} segment with alanine had no major effect on the agonist-induced internalization kinetics of the AT_{1a}-R in COS-7 cells, but replacement of Phe^{239} with alanine modestly reduced the internalization rate of the receptor (Fig. 6). Other substitutions of Ile^{238} and Phe^{239} caused impairment of the internalization kinetics of the mutant receptors (Fig. 7) that paralleled their reductions in inositol phosphate generation (Fig. 2). Replacement of Ile^{238} with alanine had no effect on the internalization kinetics, but substitution with charged residues caused a significant decrease in the internalization kinetics of the receptor (Fig. 7 and Table II). Compared with the wild-type AT_{1a}-R, the K_e values of the I238K and I238D mutant receptors were reduced to 45 ± 2% and 10 ± 0.1% of the wild-type value, respectively. The K_e values of mutant receptors in which Phe^{239} was replaced by other residues (F239K, F239R, F239Y, F239A, and F239L) were 132 ± 1.8%, 79.4 ± 0.7%, 85.8 ± 6.4%, 60.6 ± 8.3%, and 66.3 ± 8.6% of that of the wild-type receptor, respectively. In contrast, the K_e values of the F239S, F239N, and F239D mutant receptors were reduced to 4.5 ± 2.1%, 6.1 ± 3.5%, and 2.9 ± 1.0% of that of the wild-type receptor, respectively (Fig. 7 and Table II).

DISCUSSION

The N- and C-terminal portions of IL3 have been implicated in G protein coupling to several seven transmembrane domain receptors (25–27). Discontinuous sequences of IL3, IL2, and the C-terminal cytoplasmic tail have been predicted to comprise an agonist-induced conformation-dependent surface that permits interaction with G proteins (28). Earlier studies using chimeric AT_1-Rs (16) and substitutions of charged residues in the C-terminal portion of IL3 (17) suggested that this region of the receptor is also required for Ang II-induced signal generation. In addition to activating G protein-dependent second messenger systems, the AT_{1a}-R also undergoes phosphorylation and rapid internalization after agonist stimulation (29–32). Substitution of amino acids 234–240 of the AT_1-R with the corresponding sequence of the β_2 receptor impaired its internalization kinetics, suggesting that this region has a role in the agonist-induced internalization of the receptor (18). However, alanine substitutions of individual amino acids in the C-terminal region of IL3 did not affect the internalization kinetics of the AT_1-R, with the exception of the F239A mutation, which reduced the internalization kinetics in a moderate extent.

Studies on the effects of replacing the distal segment of IL3 of the AT_{1a}-R with the corresponding regions of the α_1-adrenergic, β_2-adrenergic, and AT_2 receptors revealed that the RNDDIFR sequence (from Arg^{234} to Arg^{240}) of the AT_{1a}-R has a major role in G protein coupling and receptor signaling (18). In the present work, alanine substitutions or deletions of Arg^{234}, Asn^{235}, Asp^{236}, or Asp^{237} had no significant effect on the inositol phosphate signaling or internalization kinetics of the receptor, but individual deletions of Ile^{238}, Phe^{239}, or Arg^{240}

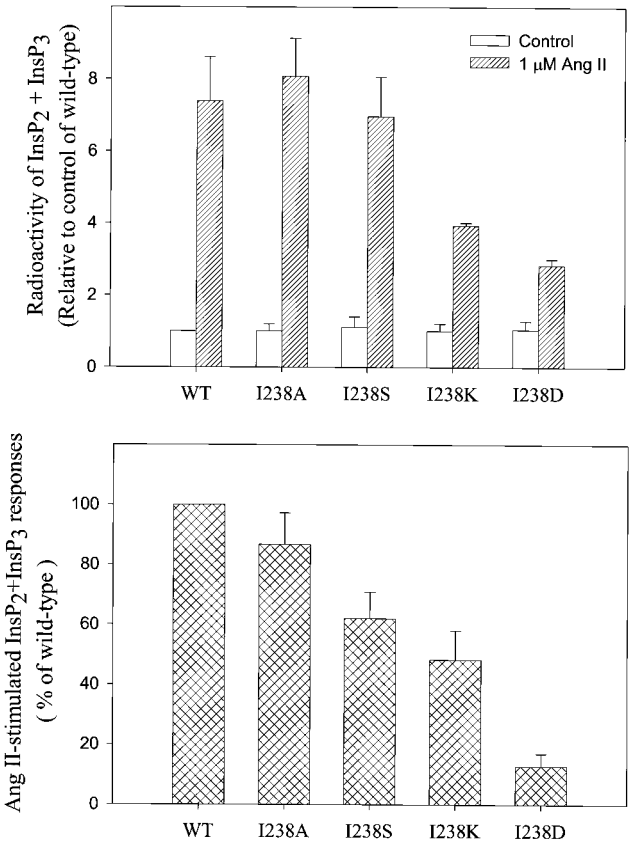

FIG. 5. **Inositol phosphate responses of Ile^{238} replacement mutant AT_{1a}-Rs.** Radioactivity of inositol bis- (*$InsP_2$*) and trisphosphates (*$InsP_3$*) (*upper panel*) and normalized inositol phosphate responses (*lower panel*) of cells expressing wild-type (*WT*) and mutant rat AT_{1a}-Rs are shown as described in the legend to Fig. 2. The data represent mean ± S.E. from three independent experiments each performed in duplicate.

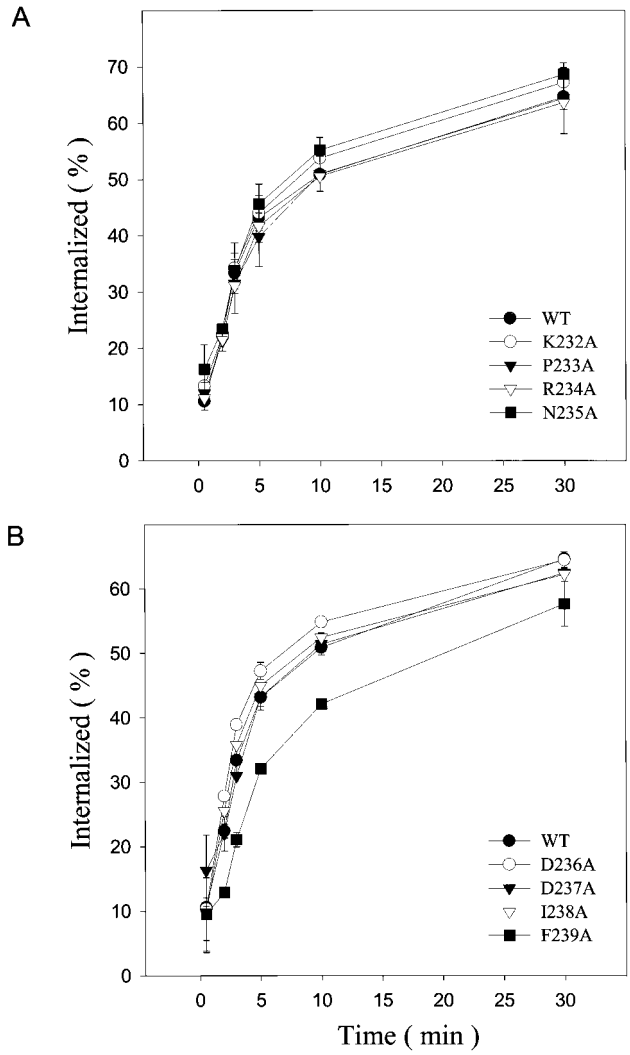

FIG. 6. **Internalization kinetics of alanine replacement mutant AT_{1a}-Rs.** COS-7 cells expressing the wild-type (*WT*) and mutant receptors were incubated with ^{125}I-Ang II at 37 °C for the indicated times. Acid-resistant and acid-sensitive binding were determined as described under "Experimental Procedures," and the internalized (acid-resistant) binding was expressed as a percentage of the total binding at each time point. Internalization kinetics of K232A, P233A, R234A, and N235A mutant AT_{1a}-Rs (*upper panel*) and that of D236A, D237A, I238A, and F239A mutant AT_{1a}-Rs (*lower panel*) are shown. The data represent mean values ± S.E. from three independent experiments each performed in duplicate.

markedly attenuated inositol phosphate production. Because the C-terminal region of IL3 is believed to form an extension of the α-helical structure of the sixth transmembrane helix, this effect of amino acid deletions could result from changes in the position of Ile^{238} relative to the rest of the helix. The lack of effect of alanine substitutions raises the possibility that, similar to the role of Leu^{222} in the N-terminal region of IL3 (33, 34), apolar amino acids in this region are favorable for signal generation.

Although substitutions with alanine or other apolar amino acids were tolerated, replacements of Ile^{238} with polar or charged amino acids significantly impaired the Ang II-induced inositol phosphate responses of the AT_{1a}-R. These data suggest that the presence of a hydrophobic amino acid at position 238 is required for AT_1-R activation. It has been reported that the four amino acid sequence, VTIL (Val^{385}, Thr^{386}, Ile^{389}, and Leu^{390}) located at the boundary of IL3 and helix 6, determines the G_i coupling specificity of the M_2 muscarinic receptor. Also, this region of the M_2 receptor has been proposed to interact with the C-terminal domain of $G\alpha_{i/o}$ (15, 35). The corresponding AALS motif (Ala^{488}, Ala^{489}, Leu^{492}, and Ser^{493}) of the G_q-coupled M_3 muscarinic receptor together with IL2 and the N-terminal part of IL3 were suggested to be directly involved in coupling to G_q (36). The position of Ile^{238} of the AT_1-R corresponds to the position of Val^{385} of the M_2 receptor and Ala^{488} of the M_3 receptor. As in the muscarinic receptors, this region appears to be an important determinant of G protein activation of the AT_1-R.

In addition to their effects on inositol phosphate signal generation, mutations of Ile^{238} also impaired the internalization kinetics of the AT_{1a}-R. Although the agonist-induced internalization of GPCRs often occurs in parallel with G protein activation, mutational analysis of the AT_1 and other receptors has shown that the structural requirements of these processes are distinct, and AT_1-R internalization can occur in the absence of inositol phosphate signal generation and G protein coupling (20, 31, 32, 37, 38). Because receptor internalization does not depend on G protein-mediated signaling, the inhibitory effects of substituting Ile^{238} with polar and charged amino acids on receptor internalization are independent of G protein coupling. Although it cannot be excluded that Ile^{238} directly couples the receptor to G protein activation and internalization, these data suggest that Ile^{238} is required for a step in receptor activation that is proximal to receptor G protein coupling. The most likely mechanism is the participation of Ile^{238} in an intramolecular interaction that is required for the active conformation of the receptor. Such a mechanism is consistent with a recent report

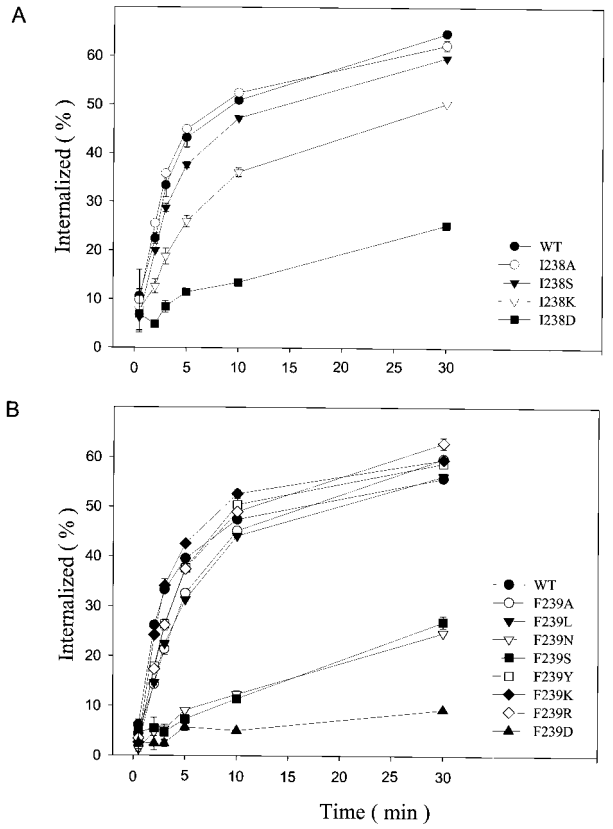

FIG. 7. **Internalization kinetics of Ile^{238} and Phe^{239} replacement mutant AT_{1a}-Rs.** COS-7 cells expressing the wild-type (*WT*) and Ile^{238} (*A*) or Phe^{238} (*B*) replacement mutant receptors were incubated with ^{125}I-Ang II at 37 °C for the indicated times. Acid-resistant and acid-sensitive binding were determined as described in Fig. 6, and the internalized (acid-resistant) binding was expressed as percent of the total binding at each time point. The data represent mean ± S.E. from three independent experiments each performed in duplicate.

TABLE II
K_e values of Ile^{238} and Phe^{239} mutant AT_{1a}-Rs

The internalization kinetics of ^{125}I-Ang II by mutant AT_1-Rs were analyzed as described under "Experimental Procedures."

AT_{1a}-Rs	K_e	*n*
	min^{-1}	
Wild-type	0.28 ± 0.04	6
I238A	0.32 ± 0.01	3
I238S	0.24 ± 0.02	3
I238K	0.13 ± 0.01	3
I238D	0.03 ± 0.00	3
F239A	0.15 ± 0.01	3
F239L	0.16 ± 0.01	3
F239N	0.02 ± 0.01	3
F239S	0.01 ± 0.01	3
F239Y	0.21 ± 0.00	3
F239K	0.33 ± 0.02	3
F239R	0.20 ± 0.01	3
F239D	0.01 ± 0.01	3

on the mapping of G protein binding domains of the AT_{1a}-R (39). In contrast to peptides derived from the N-terminal region of IL2 and IL3 and the juxtamembranous region of the cytoplasmic tail, peptides derived from the C-terminal region of IL3 did not compete for receptor G protein coupling (39). The proposed participation of Ile^{238} in an intramolecular interaction is also in agreement with the GPCR model of Baldwin (40), which predicts that the side chain of the amino acid in the position of Ile^{238} faces helix 7 and thus points away from the putative G protein binding region (28, 40).

In view of the requirement for an apolar amino acid in the position of Ile^{238} in the AT_1-R, the properties of the amino acids in this location in other members of GPCR family I were examined. Among 171 GPCR sequences from the data base of comprehensive alignment of G protein-coupled receptor sequences compiled by the Molecular Recognition Section of the National Institutes of Health, only 22 receptors contain polar amino acids at this position. These residues were 15 threonines, 4 serines, 1 asparagine, and 2 histidines. All other members of the GPCR family I contain an apolar residue at this position. The frequent occurrence of a corresponding apolar amino acid in GPCRs raises the possibility of a general requirement for such residues in GPCR activation by agonist ligands.

The position of Phe^{239} in the AT_1-R has a different amino acid requirement compared with that of position 238. The location of this amino acid corresponds to Thr^{386} in the VTIL motif of the M_2 muscarinic receptor and Ala^{489} in the AALS motif of the M_1, M_3, and M_5 muscarinic receptors. In addition, Phe^{239} corresponds to a position that frequently causes constitutive activation if substituted in adrenergic receptors (24). However, a completely different mechanism of constitutive activation has been reported for the AT_1-R (41). In contrast to Ile^{238}, replacement of Phe^{239} with polar residues had a variable effect on AT_1-R activation. Substitutions of apolar amino acids or tyrosine caused only minor changes in signal generation and internalization of the receptor, but substitution of asparagine or serine decreased these responses. Although substitution of a negatively charged aspartic acid for Phe^{239} severely impaired inositol phosphate generation and receptor internalization, replacement by positively charged residues increased these responses. Recent studies have predicted that AT_1-R activation involves an outward movement of the cytoplasmic end of the sixth transmembrane helix, accompanied by a clockwise rotation (as viewed from the cell interior) by ~30° (42). A possible explanation for the opposite effects of basic and acidic amino acids is that positively charged residues facilitate this movement, whereas the presence of a negative charge interferes with it. Similar to the results obtained with mutations of Ile^{238}, substitution of Phe^{239} caused a parallel impairment of inositol phosphate signal generation and receptor internalization. These data suggest the Phe^{239}, like Ile^{238}, has a role in the formation of the active conformation of the receptor. This conclusion is in accordance with the results of studies on rhodopsin-transducin interaction that showed that substitution of the amino acids (Val^{250} and Thr^{251}) of rhodopsin that correspond to Ile^{238} and Phe^{239} of the AT_{1a}-R interfere with the formation of the active (M II) state of the photoreceptor and thus indirectly affect its interaction with transducin (43).

In summary, this study demonstrates the importance of two specific residues, Ile^{238} and Phe^{239}, in the C terminus of IL3 in the mechanism of activation of the AT_1-R. The parallel impairment of receptor signaling and internalization suggests that these adjacent apolar residues may participate in intramolecular interaction(s) that are required for receptor activation. The exact mechanism of this interaction and its role in other GPCRs requires further investigation. However, based on the present findings, and other evidence that implicates this region of GPCRs in signal transduction, it is possible that these amino acids and their counterparts have a general role in receptor activation by agonist ligands.

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